BIOCHEMISTRY OF THE BLOOD

by
SHELDON I. GUTTMAN
Department of Zoology and Physiology
Miami University
Oxford, Ohio.

Recent findings in molecular biology show that protein structure results from the indirect translation of the genetic message encoded in part of a DNA strand. The polypeptide chain produced in this manner can be considered the primary phenotype of an individual gene. Various interactions and combinations of these polypeptide chains, at particular time intervals, produce the secondary and tertiary phenotypes of the organism and result in its morphological and physiological attributes. The more similar the genetic constitution of two organisms, the more closely they are related. From this basic observation, it follows that the degree of similarity between the proteins of organisms is directly proportional to their degree of relationship (Sibley, 1964).

Blood is a complex mixture of many components. For example, there are at least seventy distinct proteins in human plasma (Surgenor, 1954). Such a mixture can be separated into many fractions on the basis of differing solubility, molecular size and/or electric charge. Evolutionary relationships of organisms can be accurately based on comparisons of the number of fractions and the properties of each fraction.

BIOCHEMICAL TECHNIQUES

Electrophoretic and immunological techniques are the most frequently used methods of comparing the blood proteins of organisms.

I. ELECTROPHORESIS.

Fractionation of protein mixtures by electrophoresis is caused by the differential migration of the components in an electric field (Bier, 1959). Rate of migration, and therefore, distance migrated, depends upon the net charge of the protein molecule, and the resistance of the medium through which the protein moves. Net charge is determined by the dissociation of acidic and basic groups and the possible binding of buffer ions to which the proteins are exposed. If the buffer composition is specified, then the average number of charges on particular proteins is fixed; therefore, the electrical force applied to these components, under a given potential gradient, is also fixed. The mobility of the protein through the medium, then, depends upon the two opposing vectors: the electrical force and the force of frictional retardation.

Two basic types of electrophoresis have been used to examine blood proteins: A. Electrophoresis in an aqueous solution of buffer (free solution electrophoresis), B. Electrophoresis in stabilized or supporting media (zone electrophoresis).

A. Free Solution Electrophoresis.

Use of this method in evolutionary studies is limited. The cost of the basic apparatus is high and the equipment is cumbersome. Although all components can be isolated, the time required is excessive. Multiple samples cannot be examined simultaneously for direct comparison and staining procedures cannot be used. Because of these limitations, and the advantages of zone electrophoresis, discussed below, this technique will not be considered further.

B. Zone Electrophoresis.

An important advantage of this method lies in the stabilization of the migrating proteins (Bier, 1959). Migration is fixed and is relatively little influenced by heat-caused convection currents. Therefore, the required equipment is less complex so that cost can be reduced to less than ten per cent of that for free solution electrophoresis.

1. Buffer impregnated solids.

One technique utilizing buffer impregnated solids is the "hanging strip" technique which is widely accepted and is currently in use in many laboratories. In this method the filter paper, or other medium such as cellulose acetate, is either allowed to hang suspended on glass rods which function as a central support or is suspended horizontally, with a minimal amount of support near the ends of the paper (Lewis, 1960). Apparatus for the former modification of the "hanging strip" technique may be purchased from Beckman Instruments, Inc.[1]; equipment for the latter modification is manufactured by Ivan Sorvall, Inc.[2].

2. *Concentrated gels.*

Many serum proteins of vastly different molecular size have essentially equal mobilities in free solution electrophoresis and electrophoresis in buffer impregnated solids because the greater frictional retardation of the large-sized proteins often is compensated for by their greater net charge (Smithies, 1959b). In polyacrylamide and starch-gel electrophoresis the larger molecules are retarded by the small pores in the gel and, therefore, fewer blood proteins have equal mobilities. Therefore, separations are more complete. For example, Brown (1964) separated the serum proteins of *Bufo woodhousei* into eight fractions using paper electrophoresis, whereas Guttman (unpublished) resolved sixteen fractions in plasma from the same species by vertical starch-gel electrophoresis.

Smithies (1955) introduced starch-gel as a supporting medium for zone electrophoresis. The horizontal technique he described combines the advantages of zone electrophoresis with freedom from serious adsorption which is a disadvantage of electrophoresis in buffer impregnated solids. Vertical starch-gel electrophoresis (Smithies, 1959 a, b) eliminates the unstable density gradients noted in horizontal starch-gel electrophoresis. Otto Hiller[3] manufactures equipment for starch-gel electrophoresis.

Polyacrylamide offers at least three distinct advantages over starch as a supporting medium. First, polymerized acrylamide gels are flexible and have great tensile strength whereas, starch gels are relatively fragile. Second, polyacrylamide gels, when destained, are clear and can easily be scanned with a densitometer yielding quantitative information about each fraction; starch gels are transluscent and if they are made transparent, they become more brittle. Third, the preparation of hydrolyzed starch is a delicate process and the use of commercially prepared starch is recommended (Smithies, 1959b). The commercial preparation varies from one lot to another and the electrophoretic procedure must be recalibrated with each lot if the results are to be comparable. The polyacrylamide usually used consists of ninety-five per cent acrylamide and five per cent methylene bisacrylamide; because of this standard composition, recalibration for different lots is unnecessary.

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[1] Spinco Division, Beckman Instruments, Inc., Belmont, California.

[2] Ivan Sorvall, Inc., Norwalk, Connecticut.

[3] Otto Hiller, P. O. Box 1294, Madison 1, Wisconsin.

Two techniques utilizing polyacrylamide are available: disc and slab electrophoresis. Disc electrophoresis separates protein bands into layers as thin as fifty microns; these are similar to a stack of flat discs. Samples are run in small, open-ended tubes, five milimeters in diameter, one sample per tube. Many samples can be examined at the same time. Canal Industrial Corporation [4] manufactures equipment for disc electrophoresis. In slab electrophoresis many samples can be analyzed in the same gel. Components are allowed to migrate further in the thin rectangular slab of polyacrylamide than in disc electrophoresis and, therefore, greater resolution can be obtained. Larger quantities of sample can be subjected to electrophoresis in a slab than in a narrow tube; for protein isolation and purification procedures slab electrophoresis is the more efficient technique. Equipment for polyacrylamide slab electrophoresis can be obtained from E-C Apparatus Corporation [5].

A variety of histochemical procedures are available for locating specific proteins on an electropherogram. Methods are available for proteins in general and specific stains detect lipoproteins, enzymes, glycoproteins, proteins containing copper or iron, and hemoglobin, free or in combination with haptoglobins (Smithies, 1959b). Giblett, *et al.* (1959) pioneered in the use of radioactive tracers to localize iron binding proteins in concentrated gels.

When procedures for electrophoresis in concentrated gels were first introduced, the large number of observed protein zones made it necessary to correlate the results with those obtained previously by electrophoresis in buffer impregnated solids. The correlation was obtained by inserting a section of a buffer impregnated electropherogram into a gel slab adjacent to a sample of whole serum and then subjecting both samples to electrophoresis. Proteins common to the section and to the whole serum were compared. Results proved that the separation process in concentrated gels differed from that on buffer impregnated solids (Smithies, 1959b). For example, a protein which migrated in the α_1 globulin region on filter paper migrated as a prealbumin in starch gels. Some proteins which were slower than the major β-globulin component in gels, migrated faster, as α_2-globulins, on filter paper. These findings suggested that if the two processes were combined and run at right angles to each other, a more complete separation would result than if either process were used alone. The two-dimensional procedure is detailed in Poulik and Smithies (1958).

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[4] Canal Industrial Corporation, 5635 Fisher Lane, Rockville, Maryland.

[5] E-C Apparatus Corporation, 220 South 40th Street, Philadelphia, Pennsylvania.

Analysis of specific peptides can reveal evolutionary relationships. To do this, single proteins are isolated, enzymatic or non-enzymatic digests are prepared, the resulting peptide fractions are separated and characterized by a technique such as two-dimensional paper chromatography and high voltage electrophoresis or ion exchange chromatography (Sibley, 1964). Equipment for these analyses can be obtained from LKB Instruments, Inc. [6].

II. IMMUNOLOGY.

The proteins to be studied, or antigens, when injected into unrelated species stimulate the synthesis of specific serum globulins called antibodies, complementary to the introduced antigens. When the serum from an immunized animal, antiserum, is exposed to the original antigen, an antigen-antibody reaction occurs and a precipitate is produced due to the formation of macroscopic complexes (Hirschfeld, 1960).

The antigen-antibody reaction is usually allowed to proceed in a stabilizing medium, such as an agar gel, through which the antigens and antibodies diffuse. This process is called double diffusion. Wherever an immunologically homogeneous antigen-antibody system meet in optimal proportions, one or more precipitation lines appear in the medium. Ideally, the number of precipitation lines yields information regarding the number of antigens in the extract against which there are antibodies in the antiserum. Each immunoprecipitate appears to function as a selective barrier; the antigens and antibodies which have produced it cannot pass, while immunologically unrelated antigens and antibodies pass unhindered. The location of each immunoprecipitate, found between the origin of the antigens and antibodies, depends upon the concentration and the diffusibility of the components. As a result, it is probable that different complexes appear as distinct precipitation lines. The Ouchterlony technique (Ouchterlony, 1948) is the most widely used double diffusion method. Precision equipment for this method can be obtained from LKB Instruments, Inc. [7]. This technique has its limitations. A single precipitation line may be formed by several antigen-antibody complexes. In addition, when a complex protein mixture, such as serum, is examined, it is difficult to delimit the actual number of lines present (Poulik, 1960).

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[6] LKB Instruments, Inc., 4840 Rugby Avenue, Washington, D. C.

[7] Ibid.

The combination of zone electrophoresis and double diffusion antigen-antibody reactions resulted in the development of immunoelectrophoresis. This method utilizes the advantages of both techniques and eliminates some of the limitations of each. The material to be examined is first subjected to electrophoresis in a supporting medium, after which the separated components are permitted to diffuse from the supporting medium into a layer of agar. Antiserum, applied in a well located parallel to the axis of electrophoretic migration of the antigens, is allowed to diffuse perpendicularly into the same agar layer. When the antigens and antibodies meet in optimal proportions, a precipitation line results. Supplies for immunoelectrophoresis can be purchased from LKB Instruments, Inc. [8].

The interfacial, or ring test, is a simple, yet sensitive, form of the precipitin reaction (Carpenter, 1965). The antiserum and antigen are carefully layered in a small test tube with the antiserum usually on the bottom. Formation of a visible precipitin ring at the interface may occur. This method is often used to detect specific antigens.

A set of serial dilutions of antigen is mixed with antiserum in test tubes and a graph illustrating the amount of turbidity vs. antigen dilution is generated. Several antigens can be tested against the same antiserum. The area under the curves indicates serologic correspondence between the various antigens; this is a reflection of the systematic relationships of the animals from which the antigens are taken (Carpenter, *op. cit.).*

RESULTS OF
APPLICATION OF BIOCHEMICAL TECHNIQUES

Developmental and Physiological Factors Influencing Patterns.

Before evolutionary relationships based on biochemical analyses of blood proteins can be derived, the possible occurrence of variation in pattern due to developmental, sexual and physiological differences must be considered.

Studies of blood proteins in larval and metamorphosed amphibians have shown that unique patterns are possessed at each stage. Hahn (1962) noted that when paedogenic *Ambystoma tigrinum* are induced to metamorphose an albumin-like protein appears. Gasser (1964, 1967) reported that the quantity of each fraction present in the plasma of *Pleurodeles*

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[8] Ibid.

waltii and *Salamandra salamandra* increases in the metamorphosed animals. In the Ranidae, Frieden (1961) and Bennett and Frieden (1962) found that young tadpoles possess a low plasma albumin concentration. As the tadpole matures, the albumin content of the plasma increases and reaches a maximum immediately following metamorphosis. In contrast, in *Xenopus laevis* the percent albumin in the plasma continues to rise after the tadpoles metamorphose (Frieden, *op. cit.).* This same worker also noted that the hemoglobin in tadpole blood is markedly different from that found in metamorphosed frogs. In *Rana catesbeiana* Richmond (1968) found that there is an augmentation of both the number of molecular species of serum proteins and the total quantity of protein as tadpoles develop legs and metamorphose.

Sexual differences also may contribute to biochemical variation. Augustinsson (1959) found that females of the genus *Rana* possess an esterase not present in male frogs. Of the eleven species of amphibians examined by Chen (1967), one, *Bombina variegata,* appeared to show a sexual difference. In an exceptionally thorough follow-up to Chen's study, Huchon, *et al.* (1968) conclusively demonstrated, by two-dimensional electrophoresis and immunoelectrophoresis, that a β-lipoprotein is present only in the female. Rossi (1960) analyzed the serum of *Bufo bufo* thirty to sixty days after mating and found differences in protein fractions in the two sexes.

Qualitative variation of amphibian blood proteins due to changes in physiological condition has not been reported. Quantitative fluctuations, however, are common; these occur due to age (Manelli, 1958), disease (Bucovaz and Kaplan, 1957; Guttman, unpublished), starvation (Brown, 1964), and during the onset of breeding season (Cei and Bertini, 1961).

Polymorphism.

Every individual in a population is biochemically, physiologically and morphologically unique (Williams, 1956; Mayr, 1963). Before the development and utilization of electrophoresis in concentrated gels, the biochemical individuality of amphibian blood went largely unnoticed. However, Brown (1964), utilizing a limited resolution technique, found that two of eight plasma proteins in *Bufo americanus* and *B. woodhousei* varied intraspecifically.

Since the development of higher resolution techniques, individual amphibians with variant forms of blood proteins have been discovered. Hemoglobin polymorphism within the following species of amphibians has been documented: *Siren intermedia* (Guttman, 1965), *Ambystoma* *annulatum* (Newcomer, 1967), *Bufo rangeri* and *B. regularis* (Guttman, 1967), the *Bufo americanus* species group (Guttman, 1969), *Bufo arenarum* and *B. spinulosus* (Brown and Guttman, *ms)* and eleven other *Bufo* species (Guttman, *ms)*. Intrapopulation variation of the iron-binding component, transferrin, in amphibian plasma was noted in *Bufo rangeri* and *B. regularis* (Guttman, 1967), the *Bufo americanus* species group (Dessauer, *et al.*, 1962; Guttman, 1969), *B. arenarum* (Brown and Guttman, *ms)*, and fifteen other species of *Bufo* (Guttman, *ms)*. Electrophoretic patterns of whole plasma varied in single populations of *Siren intermedia* (Guttman, 1965), *Salamandra salamandra* (Gasser and Cahet, 1967), *Taricha granulosa, T. rivularis* and *T. Torosa* (Coates, 1967), and *Bufo valliceps* and *B. woodhousei* (Fox, *et al.*, 1961).

Interpopulation Variation.

Geographically isolated populations of a species vary. This geographic variation is a universal phenomenon (Mayr, 1963). Just as these populations may differ in morphological attributes, they may differ in the frequency of occurrence of particular proteins or be characterized by distinctive proteins (Dessauer and Fox, 1964). The uniqueness of local populations is a consequence of sexual reproduction. Because each individual is biochemically distinct, no two groups of organisms can be identical. If the populations occur in different environments, minor differences may become accentuated because every local population is under strong and continual selection pressure for maximal fitness in its particular habitat (Mayr, *op. cit.)*.

Prior to 1964, few findings of intra- and interpopulation variation of amphibian blood proteins were reported and workers stated that little intraspecific variation occurs within this vertebrate group (Dessauer and Fox, 1964). The papers cited in the preceding section illustrate the prematurity of this statement.

Comparisons of protein frequencies, and therefore, gene frequencies, in adjacent populations indicate the amount of interbreeding which is occurring and/or has occurred. If enough populations are examined, the direction of gene flow and evolutionary pathways may be elucidated. For instance, an approximation of the route taken by *Bufo americanus* in colonizing the islands in the upper Lake Michigan area was derived using starch-gel and gel diffusion techniques (Abramoff, *et al.*, 1964). Their data indicate that populations inhabiting the islands are serologically similar to each other and are more closely related to the populations of the eastern mainland than the western mainland. An electrophoretic examination of the transferrins and hemoglobins of thirteen populations of the widespread African toad, *Bufo regularis,* indicates that this species may be composed of two forms wich meet and exchange genes in Rhodesia and Kenya (Guttman, 1967). Coates (1967) noted that blood from the northern, southern and Sierran forms of *Taricha torosa,* showed distinctive electrophoretic patterns.

Interspecific Comparisons.

Although the three previous sections stress the great degree of variability in the blood of amphibians, the number of proteins which conspecific individuals share is usually greater than the number by which they differ. Species specific plasma patterns were reported for the following urodeles: *Ambystoma, Amphiuma, Aneides, Cryptobranchus, Diemictylus, Necturus, Plethodon, Siren, Taricha* and *Triturus* (Gleason and Friedberg, 1953; Kiortsis and Kiortsis, 1960; Dessauer and Fox, 1964; Hebard, 1964; Chen, 1967; Coates, 1967) and anurans: *Acris, Alytes, Bombina, Bufo, Hyla, Leptodactylus, Microhyla, Pleuroderma, Pseudacris, Rana, Scaphiopus* and *Xenopus* (Bertini and Cei, 1959; Cei and Bertini, 1961; Fox, *et al.*, 1961; Bertini and Cei, 1962; Barrio, 1964; Dessauer and Fox, 1964; Hebard, 1964; Chen, 1967). Chalumeau-Le Foulgoc and Gallien (1967) performed a detailed analysis of the serum proteins of two species of the salamander genus *Pleurodeles.* Their two-dimensional electropherograms demonstrated at least twenty constituents in the serum of *P. waltii* and at least twenty-one in *P. poireti* serum. The two species differ electrophoretically by five components; two of these are immunologically specific to *P. waltii.*

Interspecific Hybridization.

The blood proteins of isolated conspecific populations and populations of different species are unique. Electrophoretic studies of the proteins of hybrids, using concentrated gels, can provide evidence regarding the mechanisms of inheritance of these proteins, the amount of gene flow occurring between populations and, possibly, the role that hybridization plays in the formation of new species.

Recent studies indicate that the inheritance of particular amphibian blood proteins is under simple genetic control. Codominant, multiple alleles existing at a single locus have been suggested to be the genetic mechanism for inheritance of transferrin and hemoglobin differences found in the genus *Bufo* (Fox, *et al.*, 1961; Guttman, 1969, *ms).* An understanding of this pattern of inheritance led to a clarification of the hybridization and introgression between *Bufo rangeri* and *B. regularis* in South Africa (Guttman, 1967). Electrophoresis in concentrated gels also showed that gene exchange occurs between some members of the *Bufo americanus* species group (Guttman, 1969) and between *B. arenarum* and *B. spinulosus* (Brown and Guttman, *ms)*.

Although hybrids usually possess two components, one from each parental species, hybrid effects may occur. In some crosses, new hybrid bands are noted (Coates, 1967; Coates and Twitty, 1967; Guttman, *ms)* while in others, certain parental types are suppressed (Coates, 1967; Coates and Twitty, 1967; Brown and Guttman, *ms;* Guttman, *ms)*.

An understanding of the mechanisms of inheritance of the serum proteins in *Taricha* and the patterns possessed by each of the species enabled Coates (1967) to speculate on the evolution of the genus. By following the loss or gain of homologous components and the change in mobility of these components between species, he was able to designate the probable protein composition of an ancestral *Taricha* and develop a phylogeny.

Evidence for the evolutionary pattern within the *Ambystoma jeffersonianum* complex was reported by Uzzell and Goldblatt (1967). They noted that the diploid species *Ambystoma jeffersonianum* and *A. laterale* were each homozygous for a different component. The triploid species, *A. platineum* and *A. tremblayi,* each showed one of these bands in the homozygous condition and the second band was represented only by a single gene. From these patterns and other data, they inferred that the triploid *A. tremblayi* possesses two sets of *A. laterale* chromosomes and one set of *A. jeffersonianum* chromosomes whereas *A. platineum* has two sets of *A. jeffersonianum* chromosomes and one of *A. laterale* chromosomes.

Higher Taxa.

Electrophoresis in concentrated gels is of limited use in discerning relationships between genera or higher taxa. When this technique is used, too much detail results and broad patterns are obscured (Dessauer and Fox, 1964). Paper electrophoresis allows these patterns to be observed and thus, when higher taxa are compared, it is usually a more satisfactory tool than the high resolution methods. Homologous proteins of some genera may be located within a limited electrophoretic mobility range, patterns from some families may lack individual components and entire protein patterns of some orders may show unique mobilities.

Dessauer, *et al.* (1962) found that all genera of anurans, except *Bufo,* can be distinguished from the urodeles by the presence of transferrins which migrate slowly and which are of low concentration. They reported that the iron-binding proteins of *Bufo* are restricted in their mobility but Guttman *(ms)* showed that the mobility of toad transferrin varies greatly. Phylogenetic trends were suggested when the mobilities of hemoglobins from animals of distantly related families were compared (Dessauer, *et al.*, 1957). A tentative key to the orders of amphibians and reptiles was constructed based on plasma patterns (Dessauer and Fox, 1956) ; this key used data such as total migration, movement towards the anode, cathode or both and the presence or absence of certain fractions.

Immunological techniques can also be used to good advantage in establishing degrees of evolutionary relationship. Goodman (1964) believes that the analysis of degree of antigenic correspondence among a variety of protein homologues is an excellent method for studying the effects on the proteins of long-range evolutionary trends. Unfortunately, few researchers interested in the evolution of amphibians have explored the utilization of this technique.

Boyden and Noble (1933) used simple mixtures of serum and antiserum to obtain evidence of the relationship of *Amphiuma, Cryptobranchus, Necturus* and *Siren.* They concluded that *Cryptobranchus* is primitive and is distantly related to the other three genera, which are closely related. The same basic procedure was employed by Cei (1964) to determine the relationship of four South American families of frogs. Neotropical ranids were immunologically different from the Leptodactylidae, Hylidae and Bufonidae. *Phyllomedusa* showed strong immunological affinities to the Bufonidae and Leptodactylidae.

The use of biochemical comparisons of blood proteins to determine degrees of evolutionary relationship is just beginning. Crick (1958) stated that a vast amount of evolutionary information is contained in the amino acid sequences of the proteins of organisms. Sibley (1964) believes that, once the technique is perfected, the ultimate method of the biochemical taxonomist will make use of comparisons between complete amino acid sequences of homologous proteins. Until such a time, relatively simple electrophoretic and immunological methods will continue to furnish information of considerable evolutionary significance.

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